Spiroplasma litorale sp. nov., from Tabanid Flies (Tabanidae: Diptera) in the Southeastern United States

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Spiroplasma strain TN-1T (T = type strain), a strain serologically distinct from other spiroplasma species, groups, and subgroups, was isolated from the gut of a horsefly (Tabanus nigrovittatus) from a barrier island off the coast of North Carolina. Related strains were isolated from other Tabanus spp., T. atratus, T. americanus, T. gladiator, T. lineola, T. sulcifrons, and T. zythicolor, from coastal Georgia. Cells of strain TN-1T in culture were helical and motile with an average of 5 to 10 helical turns per cell. Electron microscopic studies determined that the cells of strain TN-1T were surrounded by a single cytoplasmic membrane, and there was no evidence of a cell wall. The spiroplasma grew well in MID and SP-4 liquid media. Serum fraction (1%) medium and conventional horse serum medium also supported growth of strain TN-1T. Fried-egg colonies were not produced; instead, the strain produced small diffuse colonies that were surrounded by satellite growth. The doubling time at the optimum temperature (32°C) was 1.6 h. No growth was observed at 5 or 43°C. Spiroplasma strain TN-1T passed through 220-nm filter pores but failed to pass through 100-nm filter pores. Strain TN-1T catalyzed glucose but hydrolyzed neither arginine nor urea. The guanine-plus-cytosine content of the DNA was about 25 ± 1 mol%, and the genome size was 1,370 kbp. Based on results from this study and previously published data, strain TN-1T (= ATCC 43211) (group XVIII) is designated the type strain of a new spiroplasma species, Spiroplasma litorale.

In 1983, one of us (R.F.W.), while visiting North Carolina’s Ocracoke Island, observed that the booth of a campground attendant was besieged, as was the entire island, with innumerable individuals of a green-eyed horsefly, Tabanus nigrovittatus (4). The attendant graciously permitted us to collect a sample of flies, from which spiroplasma strain TN-1T (T = type strain) was derived. Isolates which have similar serological profiles or which exhibit partial serological cross-reactivity with strain TN-1T were subsequently isolated from other Tabanus spp., T. atratus, T. americanus, T. gladiator, T. lineola, T. sulcifrons, and T. zythicolor collected from a barrier island off the coast of Georgia or from Bulloch County in Georgia’s coastal plain (6, 7, 22, 23). Strain TN-1T has been designated the representative strain of spiroplasma group XVIII (19). In this paper we describe the morphological, physiological, and biochemical properties of strain TN-1T (= ATCC 43211) and designate this strain the representative of a new spiroplasma species, group XVIII species Spiroplasma litorale.

MATERIALS AND METHODS

Spiroplasma strains. Strain TN-1T was isolated by standard techniques (3, 4, 12) by one of us (T.B.C.) in MID medium from the gut of a female green-eyed horsefly (T. nigrovittatus) collected at Ocracoke, N.C. Serologically related isolates were also obtained from the guts of six other tabanid fly species, T. americanus, T. atratus, T. gladiator, T. lineola, T. sulcifrons, and T. zythicolor (6, 7, 23). Some of the genomic and serological features of the organism have been reported previously (2, 19). Representative strains of all previously recognized groups (16, 17, 19, 25, 26) and subgroups, including the type strains of all previously recognized species, were also employed.

Culture medium and cultivation techniques. Spiroplasma strain TN-1T was grown in MID liquid broth (20) at 30°C. The culture was filtered and cloned (14) in MID broth containing 500 U of penicillin per ml. A triply cloned strain was designated TN-1T and was subsequently used in characterization studies. Other culture media used in the study were SF-4, conventional 20% horse serum medium, and a 1% bovine serum fraction broth (20). Solid medium was prepared by adding 2.25% Noble agar (Difco Laboratories, Detroit, Mich.). Agar cultures were incubated at 30°C either aerobically or anaerobically in a hydrogen GasPak system (BBL Microbiology Systems, Cockeysville, Md.).

Temperature studies. The temperature requirements of cultures of strain TN-1T in the late logarithmic phase in MID broth were determined as previously described (10, 11). Growth was measured at temperatures of 5, 10, 15, 20, 25, 30, 32, 37, 41, and 43°C.

Filtration. Filtration characteristics were determined in MID broth by passing logarithmic-phase cultures through filters with 450-, 300-, 220-, and 100-nm pores. Each filtrate, as well as an unfiltered control, was subcultured in a series of 12 tubes by using 10-fold dilutions and was monitored for growth (14).

Morphological studies. Cells of strain TN-1T in MID broth cultures in the logarithmic phase were examined by dark-field microscopy by using a magnification of ×1,250. Electron microscopic examination of the membrane structure of strain TN-1T by standard methods (27) has been described previously (19).

Sterol requirements. Strain TN-1T was tested for a sterol requirement by two methods; the growth obtained in a serum-free broth formulation containing various concentrations of cholesterol was assessed, and the ability of the strain to grow in serum-free or serum-containing broth media was determined.

Biochemical and physiological properties. The ability of strain TN-1T to utilize glucose, arginine, and urea was assessed by previously described methods (1, 30).

Serological tests. Antiserum to strain TN-1T was raised in rabbits as previously described (29). Immunodiffusion antiserum to all previously established groups, putative groups, and subgroups (16, 17, 19, 25, 26) of Spiroplasma species were taken from reference collections at the Beltsville Agricultural Research Center and the National Institute of Allergy and Infectious Diseases Laboratory in Frederick, Md. These antiseras and spiroplasma TN-1T were tested reciprocally in both metabolism inhibition (28) and spiroplasma deformation tests (28, 29).
was included in the serum-free broth. In experiments to test
TN-lT grew well in M1D and SP-4 broth media containing 500
Noble agar after 3 days of aerobic incubation at 30°C. Bar
organism did not replicate at 5°C, it was able to retain its
plemented with 2.25% Noble agar grown under aerobic con-
fraction. Growth occurred over a temperature range of 10 to
U of penicillin per ml. The strain also grew in conventional
brane.
organism was filamentous with no evidence of a
microscopy revealed filamentous cells with no evidence of a
Copy contained numerous helical motile filaments. Electron
Reactions were observed.
metabolism inhibition and deformation
the ability of strain TN-1T to sustain growth through 23 serial
dilutions in serum-free broth, in serum-free broth supple-
mented with 0.04% Tween 80, or in serum-containing media,
the organism showed consistent and sustained growth only in
medium containing serum. Growth was apparent only in a
single 10-fold dilution in each of the two serum-free formulations.
Spiroplasma strain TN-1T produced acid from glucose, but
no evidence of arginine or urea hydrolysis was observed.
Serological tests. Metabolism inhibition and deformation
tests performed with antisera prepared against known and
putative Spiroplasma groups and subgroups indicated that
strain TN-1T was not related serologically to representatives of
previously established groups or species or to any of the eight
representatives of putative new groups tested. No reciprocal
crosses were observed in either test. One-way crosses were
observed with 11 groups or putative groups when antiserum
against strain TN-1T was reacted with heterologous antigen
(Table 2). When strain TN-1T antigen was tested against het-
rogenous sera, a low-level one-way cross-reaction was ob-
sered with strain PALS-1 (an ungrouped representative of a
putative new group) in deformation tests. No other cross-
ctions were observed.
Genome size and DNA base composition. The genome size
of strain TN-1T was found to be 1,370 kbp by pulsed-field gel
electrophoresis (2). The base composition (guanine-plus-cy-
tosine content) of the DNA of strain TN-1T was 25.1 mol%
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Habitat. The genus Spiroplasma offers a rich opportunity to
study the ecology of microorganisms associated with insects (3,
8). Strain TN-1T described here was isolated from the gut of
the horsefly T. nigrovittatus from a barrier island, Ocracoke
Island, N.C. Strain TN-1T has not been studied as an insect
pathogen. Hundreds of spiroplasma cultures have been ob-
tained from tabanids collected on the coastal plains from Flor-
ida to Nova Scotia and in the Rocky Mountains from Montana
to New Mexico. Other sites of isolation include Vermont and
Texas. The most extensive survey was performed from 1987 to
1994 in Bullock County, Ga., some 80 km from the Atlantic
Ocean. Cultures closely related serologically to strain TN-1T
were isolated from six other Tabanus species (4-7, 22, 23).
Four isolates were obtained from T. americanus from Bullock
County, Ga., and Johnston County, N.C.; eight cultures were
obtained from T. gladiator from Bullock County, Ga.; nine
isolates were obtained from T. lineola from Bullock and Cam-
den Counties, Ga.; one isolate was obtained from T. sulcifrons
from Bullock County, Ga.; and one isolate was obtained from
T. zythicolor from Camden County, Ga. A closely related

### Table 1. Growth response of strain TN-1T to cholesterol

<table>
<thead>
<tr>
<th>Medium</th>
<th>Cholesterol concn (µg/ml)</th>
<th>Amt of protein (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1D</td>
<td>0 (Control)</td>
<td>3.06</td>
</tr>
<tr>
<td>Serum-free base medium with 1% serum fraction</td>
<td>0 (Control)</td>
<td>2.28</td>
</tr>
<tr>
<td>Serum-free base medium alone</td>
<td>0</td>
<td>0.06</td>
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<tr>
<td>Serum-free base medium with cholesterol</td>
<td>1</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.21</td>
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</tbody>
</table>

* Amount of total cell protein obtained from a cell pellet cultivated from 100 ml of each serum control medium or serum-free base medium supplemented with cholesterol.
The properties described here for strain TN-1T fulfill the proposed criteria of taxonomic nomenclature (9) for species of the class Mollicutes, including the absence of a cell wall, filterability, and penicillin resistance. The inability of this strain to utilize urea, its temperature requirements, and its helicity and motility place the organism in the family Spiroplasmataceae and the genus Spiroplasma (18). Finally, a serological comparison of strain TN-1T with other Spiroplasma species and with other unclassified spiroplasma strains that represent putative new groups demonstrated the uniqueness of the new insect strain. We therefore propose the name Spiroplasma litorale for this organism.

The taxonomic description below summarizes the properties of the organism.

**Description of Spiroplasma litorale sp. nov.** _Spiroplasma litorale_ (l.i.to.ra'le. L. neut. adj. litorale, of the shore or coastal area).

Cells are filamentous, helical, and motile, vary from 200 to 300 nm in diameter, and lack true cell walls. Colonies on solid medium containing 2.25% Noble agar are granular with dense centers, uneven margins, and multiple satellites and never have a fried-egg appearance.

Chemoorganotroph. Acid is produced from glucose. Hydrolyzes neither arginine nor urea.

Cholesterol or serum is required for growth. The temperature range for growth is 10 to 41°C, with optimum growth occurring at 32°C. The doubling time in M1D medium at the optimum temperature is 1.6 h.

Seroologically distinct from previously established _Spiroplasma_ species. Isolated from the gut of _T. nigrivittatus_ (Diptera: Tabanidae). Pathogenicity for insects has not been determined.

The guanine-plus-cytosine content of the DNA is 25 ± 1 mol%, as determined by the buoyant density method. The genome size is 1,370 kbp.

The type strain is TN-1T ( = ATCC 43211).

**ACKNOWLEDGMENT**

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**REFERENCES**

